Differential Predation on Tadpoles Influences the Potential Effects of Hybridization between *Hyla cinerea* and *Hyla gratiosa*

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Abstract.—Long-term effects of hybridization and introgression are influenced by performance of hybrids in habitats of parental species. The treefrogs *Hyla cinerea* and *Hyla gratiosa*, which typically breed in permanent and temporary habitats, respectively, have occasionally hybridized throughout the Southeastern United States. To predict in which of the parental habitats effects of hybridization might be strongest, I performed experiments to evaluate predation on tadpoles of *H. cinerea*, *H. gratiosa*, and F_1 hybrids with predators typical of the breeding habitats of the parental species. Hybrid tadpoles had lower survival with sunfish than odonate naiad (dragonfly) predators and tended to increase hiding behavior in response to sunfish predation. Tadpoles of *H. gratiosa* also had higher survival with odonates than sunfish, but *H. cinerea* had similar survival with both predator types. These results suggest that hybrids are most likely to survive and return to breed in temporary habitats used by *H. gratiosa*. Thus, hybridization and introgression might be more likely to have adverse effects on populations of *H. gratiosa* than *H. cinerea*.

Understanding factors that contribute to success or failure of hybrid individuals is critical to predicting the effects hybridization and introgression may have on parental species. If hybridization produces offspring that have equal or greater fitness than parental species, stable hybrid zones, introgression, or formation of a new species may occur (Arnold, 1997; Parris, 1999). Alternatively, lower fitness of hybrids could create selective pressures favoring reinforcement of reproductive isolation (Barton and Hewitt, 1985). In most cases, these selective pressures operate symmetrically, that is, traits that reinforce isolation are favored in each parental species.

Many studies of hybridization, including most studies of anurans, focus on hybrid zones in which species pairs co-occur across a narrow range of sympatry (Barton and Hewitt, 1985; Kruuk and Gilchrist, 1997). For these species, hybrid inferiority within the overlap zone of parental species may prevent hybrids from attaining high densities and reduce introgression (Kruuk and Gilchrist, 1997). However, in many cases, closely related species have broadly overlapping ranges but segregate by habitat type (Parris, 2001). In these cases, understanding effects of hybridization requires understanding hybrid performance in each habitat, because it is well known that relative fitness of hybrids may vary from one environmental condition to another (Parris, 2001; Parris et al., 2001). If hybrid fitness changes qualitatively between parental habitats, hybridization can have different effects in different habitats, and selective pressures on each parental species may differ.

The treefrogs *Hyla cinerea* and *Hyla gratiosa* are sister taxa that co-occur throughout the majority of their geographic ranges in the southeastern United States (Maxson and Wilson, 1975; Hedges, 1986; Lamb and Avise, 1987; Conant and Collins, 1991). Hybrids of these species have been found throughout their geographic range (Table 1), and at least one population of hybrids and backcrossed individuals has persisted for over 40 years near Auburn, Alabama. Hybrids are viable and fertile and introgression into both parental species has been documented (Mecham, 1960; Schlefer et al., 1986). Although phenotypic characteristics of F_1 hybrids including morphology and mating call are often intermediate between the parental species (Gerhardt et al., 1980; Schlefer et al., 1986), identification of backcrossed individuals using only these characteristics is impossible. In addition, use of morphological characteristics to identify hybrids significantly underestimates the proportion of hybrid and introgressive individuals in a population that are positively identified by genotypic analysis (Lamb and Avise, 1986; Lamb and Avise, 1987). Because of the difficulty of distinguishing hybrid and backcrossed individuals from either parental species, it is possible that hybridization occurs, but remains undetected by observers, at many localities throughout the range of these species. Although hybridization in these species has only been well documented at a few sites, observations of hybrids from other sites suggest that hybridization may be more widespread (Table 1).

Hybrids may form when behavioral reproductive isolation breaks down. *Hyla cinerea* and *H. gratiosa* typically breed in different aquatic habitat types: *H. cinerea* occur in permanent lakes, ponds, swamps, and occasionally temporary ponds, whereas *H. gratiosa* are restricted to temporary fishless ponds (Fig. 1; Mount, 1975). Hybridization has been documented when *H. cinerea* co-occurs with *H. gratiosa* in temporary fishless ponds (Mecham, 1960; Mount, 1975; Schlefer et al., 1986). Male *H. cinerea* typically call from emergent vegetation above the water at the edge of ponds, and male *H. gratiosa* call while floating in vegetation in deeper water. However, at habitats that lack sufficient emergent vegetation, *H. cinerea* males often call from the ground at the edge of the pond; thus, female *H. gratiosa* have a higher chance of encountering male *H. cinerea* while entering the pond (Mecham, 1960). Most cross-species mated pairs consist of male *H. cinerea* and female

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TABLE 1. Localities from which hybrid *Hyla cinerea* × *Hyla gratiosa* adult individuals have been observed or collected for research. Habitat description is listed where available either from the literature source or from the collector, NA = information not available.

Locality	Habitat description	Museum specimen	Reference
Lee Co., AL	small fishless man-made pond, no emergent vegetation	AUM 317	Mecham, 1960; G. Folkerts, pers. comm.
Calhoun Co., AL	NA	AUM 5697	NA
Jasper Co., GA	impounded creek, no emergent vegetation	NA	J. Jensen, pers. comm.
Tattnall Co., GA	natural wetland converted and deepened to a borrow pit	NA	D. Stevenson, pers. comm.
Near Savannah, GA	small man-made ponds, little emergent vegetation	NA	Gerhardt et al., 1980
Alachua Co., FL	large natural permanent lake, emergent vegetation	NA	P. Moler, pers. comm.
Leon Co., FL	small man-made drainage pond, little emergent vegetation	UF 141601	MSG
Leon Co., FL	collected near temporary ponds and roadside ditches	UF 134202	K. Enge, pers. comm.
Putnam Co., FL	NA	UF 108058	NA

H. gratiosa (Schlefer et al., 1986). Hybrid individuals are typically found at anthropogenic artificial or disturbed natural aquatic habitats in which vegetation structure has been changed (Table 1), but hybridization can also occur in natural habitats where water level fluctuations have altered vegetation structure.

The assemblage of potential tadpole predators in permanent and temporary ponds varies considerably,
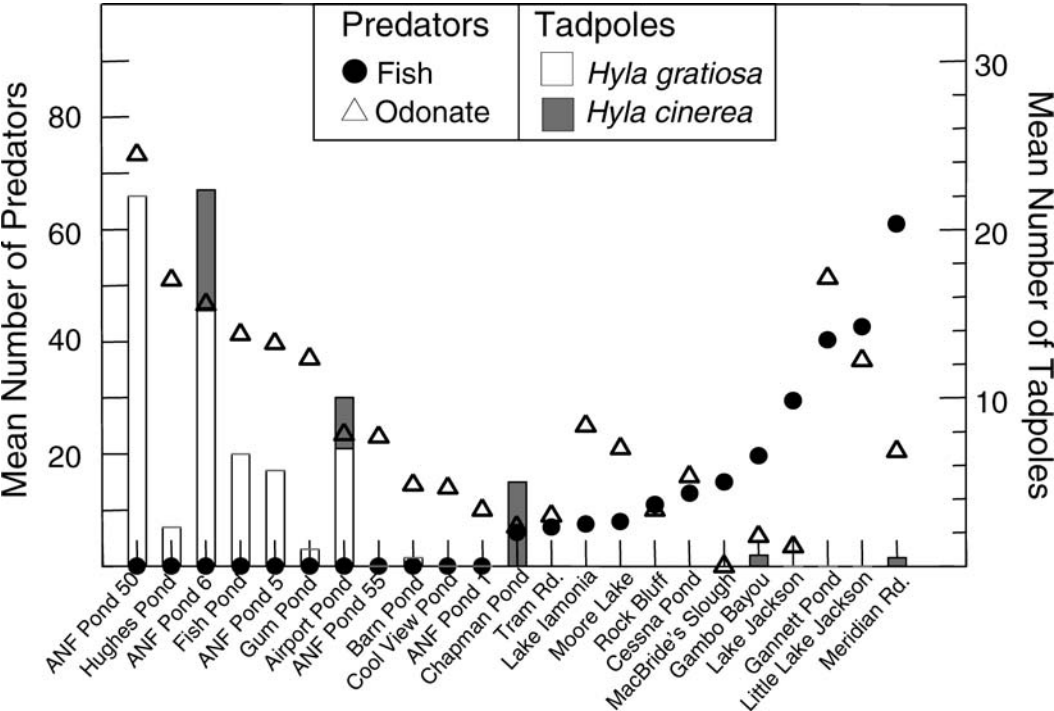

FIG. 1. Abundance of *Hyla cinerea* and *Hyla gratiosa* tadpoles (bars) and odonate naiad (Libellulidae and Aeshnidae) and fish (Centrarchidae, Poeciliidae, and Fundulidae) predators (dots), at 23 localities in northwestern Florida. Data are total number of individuals in four throws of a 0.5 m^2 box trap, averaged across sampling intervals (September 2001, May 2002, August 2002) if locality was sampled more than once (for complete sampling methods and habitat descriptions, see Gunzburger and Travis, 2004).

but fish are more abundant in permanent ponds, and, in general, odonate dragonfly naiads are more abundant in temporary ponds (Fig. 1; Gunzburger, 2004; Gunzburger and Travis, 2004). Predation on the tadpole stage may influence the habitats in which *H. cinerea*, *H. gratiosa*, and their hybrids occur (Gunzburger and Travis, 2004). One previous study demonstrated that larval *H. gratiosa* suffer higher predation rates with Bluegill Sunfish (*Lepomis macrochirus*) than larva of *H. cinerea* and hybrids (Blouin, 1990). Differential survival of hybrids under different predatory regimes may result in asymmetric patterns of introgression into the parental species.

The objective of this study was to evaluate the role of tadpole predation on the ability of hybrid tadpoles to survive in the habitats of the two parental species. I evaluated the survival of *H. cinerea*, *H. gratiosa*, and hybrid tadpoles and behavior of hybrid tadpoles in response to two predator species: a fish typical of breeding sites of *H. cinerea* (Warmouth Sunfish, *Lepomis gulosus*), and a dragonfly naiad typical of breeding sites of *H. gratiosa* (aeshnid odonate naiad, *Anax junius*). For this study, I chose to evaluate warmouth because they prefer larger prey, such as small fish and, thus, are more likely to prey on tadpoles in nature than are Bluegill Sunfish, which consume primarily plankton and aquatic insects (Boschung and Mayden, 2004).

MATERIALS AND METHODS

Tadpole Survival Experiment.—The first experiment evaluated survival of F_1 hybrid larva of *H. cinerea* × *H. gratiosa* relative to larva of each parental species with predators typical of breeding habitats of *H. cinerea* and *H. gratiosa*. This experiment consisted of six treatments: three tadpole types (*H. cinerea*, *H. gratiosa*, or hybrid tadpoles) crossed with two predator types (warmouth or odonate). I performed three replicates of treatments with hybrid tadpoles and four replicates of treatments with *H. cinerea* and *H. gratiosa*. This experiment was performed in 22 large cattle trough mesocosms filled with well water and arranged in three rows in a field at the Florida State University greenhouse facility in Tallahassee, Florida. The troughs were $183 \times 61 \times 61$ cm (approximate total volume 568 liters), with a depth gradient from 5–50 cm water created by piling sand at one end, and covered with tight-fitting plastic window-screen lids. Refuges constructed of sliced plastic garbage bags weighted with rocks were placed throughout each mesocosm (Gunzburger, 2004). Treatments were randomly assigned to troughs, and to reduce any potential spatial effects, I adopted a rule that no more than two replicates of the same treatment were allowed in any one row and replicates of the same treatment had to be separated by at least one trough.

I collected amplexed pairs of adult frogs on 17 May 2003 to provide eggs to raise into tadpoles. Five amplexed pairs of *H. cinerea* were collected from Innovation Pond (30.427N, 84.324W) and eight *H. gratiosa* were collected from Kit Pond (30.375N, 84.371W), both in Leon County, Florida. To form hybrids, I selected two pairs each of *H. cinerea* and *H. gratiosa* and separated each pair at 0100 h. Then I paired individuals of the opposite sex and different species in small (1 liter) plastic containers with ventilation holes in the top. All four pairs entered amplexus within 15 min. To collect eggs, I placed each pair of

amplexed frogs in a separate covered bucket with well water overnight for oviposition. Three of the four hybrid pairs oviposited eggs in the water. One pair deposited eggs on the side of the bucket above water level; these eggs did not develop and were not used for the experiment. Thus, for the experiment, I used tadpoles raised from three *H. cinerea*, six *H. gratiosa*, and three hybrid egg clutches. Eggs were raised until hatching for two days in the buckets in which they were oviposited; each bucket was aerated with an airstone and partial water changes were performed daily. Then hatchling tadpoles were pooled by type and transported to the greenhouse facility and raised to the appropriate size for the experiment in three large (1.2 m diameter, 0.7 m deep) outdoor holding tanks. These tanks had tight-fitting plastic windowscreen lids and contained algae as a food source for the tadpoles, a diet that I supplemented with daily additions of ground rabbit chow and TetraMin® fish flakes.

Predators were collected from two ponds in Leon County, Florida: odonates were collected by dipnet from ANF Pond 50 (30.347N, 84.322W), and warmouth were collected by seine from Trout Pond (30.334N, 84.387W). Predators were maintained in the greenhouse facility in small plastic containers (odonates) or aerated aquaria (warmouth) prior to use in experiments. Warmouth generally have faster digestion rates than odonates (pers. obs.); thus, in an attempt to control hunger level of predators, warmouth were starved for 18 h and odonates were starved for three days prior to use in an experiment. On the first day of the experiment (3 June 2003), I haphazardly selected 40 tadpoles to add to each of the 22 troughs. A subset of tadpoles was measured prior to the experiment by photographing them in a small tray with a ruler. Although tadpoles were the same age (17 days postoviposition), both the *H. gratiosa* (mean total length [TL] = 14 ± 2.4 mm) and hybrid tadpoles (13.3 ± 2.8) were larger than *H. cinerea* tadpoles (9.2 ± 1 mm; ANOVA, $F_{2,28} = 14$, $P < 0.001$). After allowing tadpoles to acclimate to the troughs, I randomly assigned and added one warmouth (mean fork length = 42 ± 6.5 mm) or one odonate (mean total body length = 26 ± 2.8 mm) to the appropriate troughs in the morning of the fourth day (6 June 2003). The experiment ended on the sixth day (8 June 2003) when all predators and surviving tadpoles were removed from each trough and the number of surviving tadpoles was counted. Each tadpole and predator individual was used only once for this experiment. All predators and surviving tadpoles of *H. cinerea* and *H. gratiosa* were released at the site of capture. Hybrid tadpoles were sacrificed by overdose of MS-222.

I used ANOVA to determine whether the proportion of tadpoles surviving varied across predator species and tadpole type. Data were arcsine-square-root transformed prior to analysis. I performed post hoc tests using Bonferroni correction for multiple tests. Two outliers were identified by residual analysis and were removed from the data: a *H. gratiosa* with warmouth replicate with 0.78 survival, and a *H. cinerea* with odonate replicate with 0.98 survival. All statistical tests were conducted using SYSTAT software.

Hybrid Behavior Experiment.—The second experiment evaluated the antipredator hiding behavior of hybrid tadpoles in response to warmouth and odonate predators. I evaluated the proportion of tadpoles
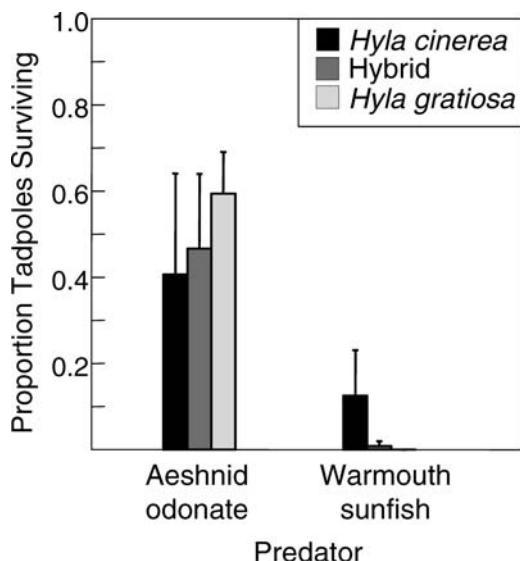
FIG. 2. Proportion *Hyla cinerea*, *Hyla gratiosa*, and F₁ hybrid tadpoles surviving with aeshnid odonate naiad (*Anax junius*) and warmouth sunfish (*Lepomis gulosus*) predators. Bars are mean with SE.

hiding before and after exposure to predation and the proportion tadpoles surviving. This experiment was performed using 1-m diameter wading pools filled to a depth of 12 cm with well water (approximate water volume 94 liters). Each pool had two refuges: a folded mesh structure ($53 \times 23 \times 12$ cm, 20 mm mesh) to mimic aquatic vegetation, and flat mesh pieces (30×30 cm, 5 mm mesh) that covered approximately 50% of the bottom of the pool to mimic leaf litter (Gunzburger and Travis, 2004). I performed four replicates of each of the two predator treatments.

I collected two amplexed pairs of *H. cinerea* from Harriman Pond (30.476N, 84.252W) and two amplexed pairs of *H. gratiosa* from Rivers Road Pond (30.369N, 84.328W), both in Leon County, Florida on 29 July 2003. I separated these pairs and formed four cross-species mated pairs as described above. Two of these pairs produced fertilized eggs that I raised into tadpoles as described above for the first experiment. Hybrid tadpoles were raised in the large stock tanks until 19 days after oviposition (mean TL = 31 ± 2.9 mm). Predators were collected and maintained at the greenhouse as described above. I began this experiment on 17 August 2003 when I placed 30 tadpoles in each wading pool at 1045 h. On 18 August 2003 I randomly selected and added one warmouth (mean fork length = 43 ± 2.5 mm) or one odonate (mean total body length = 26 ± 2.6 mm) to the appropriate pools at 1045 h. Immediately after I added the predator, I counted the number of tadpoles visible outside the mesh refuges in each pool. After 48 h of exposure to predation, on 20 August 2003, I again counted the number of tadpoles visible outside the refuges and then removed predators from each pool and counted the number of surviving tadpoles.

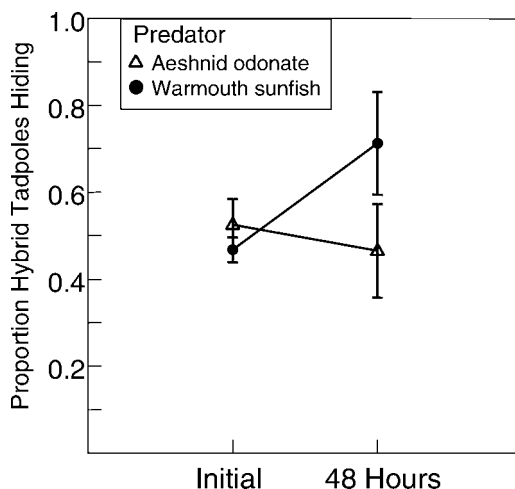I compared tadpole survival in the two predator treatments with a *t*-test. Then I evaluated whether hybrid tadpoles respond differently to the two predator types by comparing the arcsine-square-root transformed proportion tadpoles hiding at the beginning and the end of the experiment using an ANOVA with predator type and time interval (initial or after 48 h) as factors.

FIG. 3. Proportion F₁ hybrid *Hyla cinerea* × *Hyla gratiosa* tadpoles hiding initially and after 48 h of exposure to predation by aeshnid odonate naiad or warmouth sunfish. Dots are mean with SE.

RESULTS

Tadpole Survival Experiment.—Tadpole survival was higher with odonates than warmouth (ANOVA, $F_{1,14} = 43.7$, $P < 0.001$; Fig. 2). For each predator type, there was a trend for tadpoles that typically occur with that predator in nature to have the highest survival; hybrid tadpoles had intermediate survival, and tadpoles that usually do not occur with that predator type in nature had the lowest survival (Fig. 2). Survival of hybrid tadpoles with warmouth was extremely low, and survival of *H. gratiosa* with warmouth was zero in the three remaining replicates after one outlier was removed (Fig. 2). There was a significant interaction of predator and tadpole type on tadpole survival (ANOVA, $F_{2,14} = 5$, $P = 0.024$). Post hoc tests indicated that survival of *H. gratiosa* with odonates was significantly higher than all tadpole treatments with warmouth, and survival of hybrid with odonates was higher than survival of hybrid and *H. gratiosa* tadpoles with warmouth (Fig. 2). Survival of *H. cinerea* did not differ significantly between warmouth and odonate predators.

Hybrid Behavior Experiment.—Survival of tadpoles was very high in this experiment (98% with odonates and 95% with warmouth) and did not differ for the two predator types (*t*-test, $t = 0.93$, df = 3, $P = 0.39$). Hybrid tadpoles in this experiment were significantly larger (twice as large) than tadpoles in the Tadpole Survival Experiment (*t*-test, $t = -16.4$, $P < 0.001$). Thus, most tadpoles had apparently grown beyond the size range of significant threat from these predators. Despite extremely low predation rates, hybrid tadpoles varied in their behavioral response to odonate and warmouth predators (Fig. 3). A marginally significant interaction

effect between predator and time interval (ANOVA, $F_{1,12} = 4.46$, $P = 0.056$) suggests that, initially, a similar proportion of tadpoles were hiding in both predator treatments, but after 48 h, more tadpoles were hiding with warmouth than odonates (Fig. 3).

Discussion

The objective of this research was to evaluate whether $H.$ $cinerea \times H.$ $gratiosa$ hybrids had differential survival under the tadpole predator regimes typical of the two parental species (Warmouth Sunfish for $H.$ $cinerea$ and aeshnid odonate naiads for $H.$ $gratiosa$). The Tadpole Survival Experiment demonstrated that both hybrids and $H.$ $gratiosa$ tadpoles are unlikely to survive in habitats with Warmouth Sunfish predators (Fig. 2). In the Hybrid Behavior Experiment, large-sized hybrid tadpoles tended to increase their hiding behavior in response to the presence of Warmouth Sunfish. However, despite this hiding behavior, smaller-sized hybrid tadpoles had very low survival with fish, indicating that hiding does not provide complete protection from warmouth predation. In addition, hiding behavior may vary across tadpole sizes. Other tadpole behaviors not evaluated in this experiment, such as reduction of activity level, may play a role in this predator-prey interaction (Werner, 1991). My results are in striking contrast to Blouin (1990) who found that with a bluegill sunfish predator, hybrid tadpoles had similar survivorship to $H.$ $cinerea$ and significantly higher survival than $H.$ $gratiosa$. I found that hybrid tadpoles had similarly low survival rates as $H.$ $gratiosa$ with Warmouth Sunfish predators. Differences in the relative sensory perceptions or feeding rates of these two species of sunfish used as predators might account for the different predation rates.

With an aeshnid odonate predator, hybrid tadpoles did not increase hiding behavior and survived almost as well as tadpoles of $H.$ $gratiosa$. This result is concordant with the distribution of adult hybrids in nature, which are generally found at temporary ponds (Table 1). Although nothing is known about the habitat distribution of hybrid tadpoles in nature, they probably do not occur with fish predators. Formation of hybrids in permanent habitats is unlikely because adults of $H.$ $gratiosa$ consistently avoid permanent habitats with fish (Mount, 1975), probably because survival of larval $H.$ $gratiosa$ with fish is almost zero (Fig. 2). If hybrid eggs were oviposited in a pond that contained fish, this experiment suggests any resultant tadpoles would have very low survival rates to metamorphosis (Fig. 2). Because this study only evaluated F_1 hybrids, it is impossible to predict the survival of F_2 and backcrossed tadpoles (Parris et al., 1999). Backcrossed and F_2 hybrid $H.$ $cinerea \times H.$ $gratiosa$ adults have been found at the most well-studied hybrid population near Auburn, Alabama (Schlefer et al., 1986; Lamb and Avise, 1987). This study did not demonstrate that hybrid tadpoles have higher survival than either parental species with the permanent pond or temporary pond predator; thus, it is unlikely that hybrids have higher fitness than the parental species in either habitat type (Parris, 1999).

Hybrid adults are probably capable of dispersing to permanent ponds, but some amphibian species return to breed in their natal pond, suggesting that hybrids may be more likely to return to breed in temporary ponds (Gill, 1978). Also, adult treefrogs may select potential breeding habitats based on the likelihood of their offspring surviving with the community of larval predators, and thus hybrids may avoid permanent habitats (Resetarits and Wilbur, 1991). Therefore, it seems likely that most hybrids will breed in temporary ponds, leading to introgression into populations of $H.$ $gratiosa$. Survival of hybrid tadpoles in permanent ponds will be very low, resulting in little if any persistent introgression into populations of $H.$ $cinerea$ at permanent ponds. The differential predation on tadpoles demonstrated in this study is a potential mechanism to explain the prevalence of observations of hybrids at temporary, fishless ponds (Table 1; Mecham, 1960; Schlefer et al., 1986).

Currently, populations of both $H.$ $cinerea$ and $H.$ $gratiosa$ are considered stable throughout their ranges and are not known to be in decline (Young et al., 2004). However, hybridization could potentially cause declines in populations of $H.$ $gratiosa$ because it may act synergistically with the effects of habitat loss and alteration (Schlyter et al., 1991). Small, isolated wetlands suitable for $H.$ $gratiosa$ are at high risk for destruction or alteration through deepening, vegetation alteration, and the addition of fish predators (Semlitsch and Bodie, 1998; Whitney et al., 2004). In another system of amphibian hybridization, habitat alteration resulted in an increased proportion of hybrids between native and introduced Tiger Salamanders in California (Riley et al., 2003). It seems likely that with increasing habitat alteration, populations of $H.$ $gratiosa$ may decrease at the landscape level as populations of $H.$ $cinerea$ increase, because many human-made or altered habitats are suitable for $H.$ $cinerea$ breeding. An increase in habitat availability for $H.$ $cinerea$ and habitat alteration of breeding ponds of $H.$ $gratiosa$ may result in increasing opportunities for hybridization between these species.

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